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The infection of reintroduced ruminants – *Bison bonasus* and *Alces alces* – with *Anaplasma phagocytophilum* in northern Poland

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Abstract

The north-eastern part of Poland is considered an area of high risk for infection with tick-borne diseases, including with human granulocytic ehrlichiosis (HGE) agents. The etiological agent of HGE is *Anaplasma phagocytophilum*. As the animal reservoir for *A. phagocytophilum* in the environment serve the species from Cervidae and Bovidae families. European bison (*Bison bonasus*) and elk (*Alces alces*) are the big ruminant species, reintroduced to the forests of Middle Europe after many decades of absence. In the foci of zoonotic diseases they are able to play a role as natural reservoir to pathogens, however, their status as protected animals means their study has been rare and fragmentary. The studies of *B. bonasus* were conducted in Białowieża Primeval Forest and *A. alces* in Biebrza National Park. PCR amplifications were performed using primers amplifying the end of the *groES* gene, the intergenic spacer and approximately two-thirds of the *groEL* gene in the first round, and primers that span a 395-bp region of the *groEL* gene were used in the second round. The positive results were obtained in *B. bonasus* and *A. alces*, the prevalence of infection was 66.7 and 20.0%, respectively. Randomly selected samples were sequenced, sequences were compared with GenBank entries using Blast N2.2.13 and determined as *A. phagocytophilum*. The results presented herein are the first record of the presence of *Anaplasma phagocytophilum* in *A. alces*, and at the same time confirm the previous observations regarding the infection of *B. bonasus* with *A. phagocytophilum*.

Keywords

Anaplasma phagocytophilum, *Bison bonasus*, *Alces alces*

Introduction

The north-eastern part of Poland is considered an area of high risk for infection with tick-borne diseases, including with human granulocytic ehrlichiosis (HGE) agents. The etiological agent of HGE is *Anaplasma phagocytophilum*. It is Gram(-) bacteria, belonging to Anaplasmataceae family. It is obligatory parasite, which grows and replicates in the membrane-bound vacuoles of vertebrate and invertebrate host's cell (Dumler *et al.* 2001). *A. phagocytophilum* has been discovered for the first time in European ruminants in 1932. For over 70 years it was considered as pathogen of domestic and free living ruminants in Europe (Woldehiwet 2006). Human infections with *A. phagocytophilum* were detected between years 1990-1994 in the USA for the first time (Chen *et al.*

1994). In Poland, the first case of HGE infections was diagnosed by PCR analysis in 2001 (Tylewska-Wierzbowska *et al.* 2001). As the animal reservoir for *A. phagocytophilum* in the environment serve the species from Cervidae family. In Europe the following species are: roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), elk (*Alces alces*). In Poland, *A. phagocytophilum* was also detected in the blood of the European bison (*Bison bonasus*) free living in Białowieża Primeval Forest (Petrovec *et al.* 2002; Stuen *et al.* 2002; Grzeszczuk *et al.* 2004b).

The most important reason for the high risk of infection is exposure to ticks, which is linked with an observed increase in the tick population, and the observed spread of ticks to new geographic areas (Gage *et al.* 2008). The main factor significantly favoring tick expansion and the in-

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crease in the incidence of tick-borne diseases is global warming, while another factor is the growth of the big ruminant populations as well as small mammal populations which are important tick hosts. Over recent years another factor has become important – this is the reintroduction of large herbivorous animals to their natural environment, which were for many years absent in the free-living state. In Poland such species are the European bison (*Bison bonasus*) and elk (*Alces alces*). The restitution of *B. bonasus* began in 1929 in Białowieża, based on the release of individuals purchased from zoological gardens. In 1952, bison were reintroduced on wild grounds of the Polish part of the Białowieża Forest, as the beginning of the present free ranging population (Kraśńska and Kraśński 2007). Elk were absent in middle-European forests during the first half of the 20th century, and returned to Białowieża Primeval Forest and other national parks in 1970's (Pucek and Raczyński 1983). These big ruminants are hosts for ticks, and consequently can be infected with tick-transmitted pathogens, however, due to their status as protected animals, the study of their roles as reservoirs for babesiosis, borreliosis, HGE and other tick-borne pathogens is difficult and still fragmentary (Grzeszczuk *et al.* 2004a; Karbowski *et al.* 2004, 2014).

Materials and Methods

The studies of European bison were conducted in Białowieża Primeval Forest (N52°29'–52°57', E23°31'–24°21'), and the studies of elk in Biebrza National Park (N53°48', E21°34'). The blood samples were obtained from legally hunted animals. Material used in the study was gathered during 2009–2011. Blood specimens were collected into sterile, EDTA containing microtubes and stored at 4°C prior to DNA isolation. To sum up, the blood samples were obtained from 5 elk and 36 bison. DNA isolation was conducted using the NucleoSpin® Blood Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. All DNA samples were eluted in 50 µl of elution buffer and stored at –20°C for further analyses.

PCR amplification

In order to verify whether genomic DNA had been successfully isolated from each blood sample, PCR amplification of a 145-bp long orthologous fragment of the vertebrate mitochondrial 12S rRNA gene was performed as published previously by Humair *et al.* (2007). 12S rRNA is a housekeeping gene present in the genome of all vertebrates. Subsequently, only 12S positive samples were further analyzed for the presence of pathogens. All samples were tested with a nested PCR targeting the *groESL* gene of *A. phagocytophilum* by using the primers HS1 and HS6 in the first round. These primers amplify the end of the *groES* gene, the intergenic spacer and approximately two-thirds of the *groEL* gene (Sumner *et al.* 1997; Lotric-Furlan *et al.* 2001). In the second round, HSVF and

HSVR primers that span a 395-bp region of the *groEL* gene were used (Lotric-Furlan *et al.* 1998).

All PCR amplifications were performed in a total of 25 µl of reaction mixture HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany), containing 2.5 µl of 10 × PCR Buffer, 1 µl of MgCl₂, 0.5 µl of dNTP Mix, 0.5 µl of each primer, 0.125 µl of HotStarTaq Plus DNA Polymerase, 14.875 µl of deionized sterile water, and 5 µl of DNA template. Nuclease free water was added instead of DNA as negative control in each assay. As positive control, previously sequenced *A. phagocytophilum* DNA was used. PCR reactions were performed in a thermal cycler (Bio-Rad) and PCR products were analyzed electrophoretically with a 1.5% agarose gel stained with GelRed stain and further visualized under UV light. DNA isolations and PCR mix preparations were conducted in separate rooms to avoid contamination.

Sequencing

Randomly selected samples were sequenced. Sequence analyses of PCR products were performed using internal primers HSVR or HSVF at the Department of Molecular Biology (Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia). The complementary strands of sequenced products were manually assembled using MEGA 4.1 software. Sequences were compared with GenBank entries using Blast N2.2.13 and determined as *A. phagocytophilum*. The nucleotide sequence of the *groEL* gene isolated from European bison, with a length of 388-bp, was submitted to the GenBank database under the accession number JN935924. Unfortunately, we did not obtain the nucleotide sequence from *Anaplasma* – positive elk.

Results

In total, 24 out of 36 samples of DNA isolated from European bison and one of elk tested positive for the presence of *A. phagocytophilum*. The prevalence of infection reached 66.7%. The *A. phagocytophilum* nucleotide sequences from bison were compared with sequences from GenBank and showed 100% identity in the overlapped region with the *A. phagocytophilum* L6-9 isolate obtained from an *Ixodes ricinus* nymph from Spain (HM057233).

Discussion

Anaplasma phagocytophilum, a rickettsial pathogen, the agent responsible for granulocytic anaplasmosis of humans and animals, is widely distributed in the natural environment in the Northern Hemisphere. The bacterium is transmitted from one host to another by the bite of infected ixodid ticks – in Europe *Ixodes ricinus* (Grzeszczuk *et al.* 2004a; Silaghi *et al.* 2008; Sytykiewicz *et al.* 2012) and possibly *Ixodes trianguliceps* (Bown *et al.* 2003), in Northern America *Ixodes scapularis*

(Morissette *et al.* 2009), *Ixodes pacificus*, *Dermacentor variabilis*, *Dermacentor occidentalis* (Holden *et al.* 2003) and *Dermacentor albipictus* (Baldrige *et al.* 2009). In Europe as well as in the USA the bacteria has been detected in a wide range of studied animals; the reservoirs of the bacteria include big, medium and small-sized mammals (Grzeszczuk *et al.* 2006; Karbowski *et al.* 2009; Hapunik *et al.* 2011).

The results presented herein are the first record of the presence of *A. phagocytophilum* in the elk (*A. alces*), and at the same time confirm the previous observations of Grzeszczuk *et al.* (2004b) regarding the infection of *B. bonasus* with *A. phagocytophilum*. Grzeszczuk *et al.* (2004b) used different primer pairs, amplifying portion of 16S rDNA of *A. phagocytophilum*: ge3a and ge10r, and ge9f and ge2 for the second round reaction, and they observed similarly high prevalence of infection, which reached 62.5%.

European bison and elk are considered animals under threat of extinction and they are strongly protected. Especially for bison, many years of inbreeding and low genetic differentiation means that each pathogen is a potential threat for the conservation of the species (Kraśńska and Kraśński 2007). This study confirms the high rate of infection in the bison population, which together with the presence of another naturally-infected animal, produces reservoirs for anaplasmosis agents in the natural environment that may result in a threat for humans (professionals – foresters, as well as tourists).

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